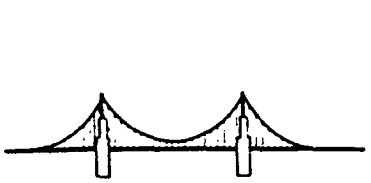

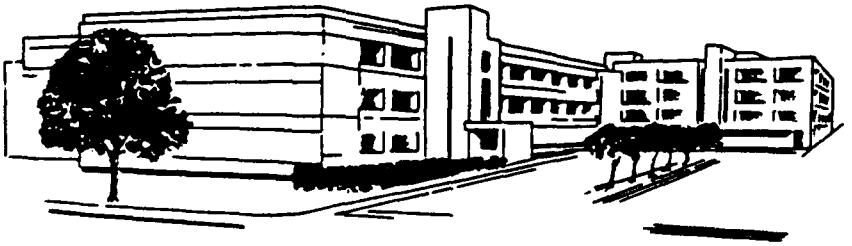

INSTITUTE REPORT NO. 480

THE RELATIONSHIP OF METALLOTHIONEIN INDUCTION TO THE HEAT SHOCK RESPONSE

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April 1993

LETTERMAN ARMY INSTITUTE OF RESEARCH PRESIDIO OF SAN FRANCISCO CALIFORNIA 94129

THE RELATIONSHIP OF METALLOTHIONEIN INDUCTION TO THE HEAT SHOCK RESPONSE -- J.M. Yang et al

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3 May 93

John R. Hess (date)
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Commander

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; Distribution is UNLIMITED.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) Institute Report # 480	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Ocular Hazards Division 6b. OFFICE SYMBOL (If applicable) SGRD-UWB-L	7a. NAME OF MONITORING ORGANIZATION U.S. Army Medical Research and Development Command
6c. ADDRESS (City, State, and ZIP Code) Letterman Army Institute of Research Bldg 1110 Presidio of San Francisco, CA 94129-6800	7b. ADDRESS (City, State, and ZIP Code) Ft. Detrick Frederick, MD 21701-5012
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 43002 / PROJECT NO. D819 / TASK NO. 891/AA / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
The Relationship of Metallothionein Induction to the Heat Shock Response

12. PERSONAL AUTHOR(S)
J.M. Yang, P.D. Bowman, M.A. Deaton, S.T. Schuschereba, B.E. Stuck

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM _____ TO _____	1993 April	39

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Sodium arsenite, heat shock proteins, stress points

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Heat shock proteins (hsps) and Metallothioneins (MTs) are sets of proteins specifically synthesized by cells in response to sublethal injury such as heat, heavy metals, or radiations. This dissertation investigated the relationship of metallothionein induction to the heat shock response. Sodium arsenite, which induces both hsps and MTs was used to establish a model system for investigation. Electrophoresis conditions that allowed simultaneous identification and quantitation of hsps and MTs were then developed. Identification of MTs was achieved by [^{35}S] cysteine and methionine labeling and [^{109}Cd]-binding. The results of this study indicate that MTs undergo increased synthesis following both heat shock and sodium arsenite treatment. The level of increase of MTs after heat shock was about one-tenth of the increase after sodium arsenite treatment. These results suggest that MTs are heat shock and stress proteins.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL John R. Hess, COL, MC, Commanding	22b. TELEPHONE (Include Area Code) (451) 561-3600 22c. OFFICE SYMBOL SGRD-ULZ

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

ABSTRACT

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Sodium arsenite, which induces both hsps and MTs was used to establish a model system for investigation. Electrophoresis conditions that allowed simultaneous identification and quantitation of hsps and MTs were then developed. Identification of MTs was achieved by [^{35}S] cysteine and methionine labeling and [^{109}Cd]-binding

The results of this study indicate that MTs undergo increased synthesis following both heat shock and sodium arsenite treatment. The level of increase of MTs after heat shock was about one-tenth of the increase after sodium arsenite treatment. These results suggest that MTs are heat shock and stress proteins.

FOREWORD

The research effort reported here served as a thesis submitted by the senior author to the faculty of the Cell and Molecular Biology Department at San Francisco State University in partial fulfillment of the requirements for a Master of Arts degree in Cell and Molecular Biology.

THE RELATIONSHIP OF METALLOTHIONEIN INDUCTION TO THE HEAT SHOCK RESPONSE -Yang et al.

HEAT SHOCK PROTEINS

Heat shock proteins (hsps) are ubiquitous polypeptides whose syntheses increase in response to sub-lethal thermal shock or a variety of other stimuli [1]. The initial observation of the phenomenon, now known as the heat shock response, or stress response, occurred in 1962 when Ritossa [2] subjected *Drosophila* embryos to thermal stress. Microscopically, Ritossa observed the formation of particular chromosomal puffs indicating specific gene expression. A biochemical correlate to this observation was made in 1974 by Tissieres et al. [3] who found that sublethal heat shock caused increases in the syntheses of specific proteins. Proteins displaying increased rates of synthesis following heat shock are defined as heat shock proteins (hsps), and each is identified by its molecular weight (MW) on an SDS-PAGE gel. Hsps are also referred to as stress proteins because a variety of other stimuli have been found to increase their syntheses [4, 5]. Hsps have been found in virtually all cells and organisms studied [1]. The response and the MWs of the associated proteins remain fairly constant between prokaryotes and eukaryotes, suggesting fundamental function(s) (reviewed in [1, 6]). Although the precise function of heat shock proteins is not entirely understood, studies have shown that basal levels of hsps exist in unstressed cells, and that their presence is vital to cell survival [7].

Increased levels of hsps bestow increased tolerance against subsequent stressors such as heat, ionizing and non-ionizing radiation, and against certain toxic chemicals [1]. Decline in tolerance to such agents parallels decreases in intracellular heat shock protein concentrations [7]. Thus, it appears that the heat shock response may buffer cells against small changes in their microenvironment [8].

METALLOTHIONEIN

The Metallothioneins (MTs) are intracellular polypeptides that have a somewhat different role in cell metabolism. MTs are ubiquitous, cysteine-rich, low molecular weight polypeptides [9]. They were initially described as cadmium-(Cd) and zinc-(Zn) binding proteins [10]. Subsequently, MTs have been identified in a broad range of eukaryotic species as well as in some prokaryotic cells. Mammalian MT is most abundant in the parenchyma of liver,

kidney, and intestine, but all cells seem to have the ability to synthesize them [11]. Although some interspecies variations exist, all mammalian MTs are 61- or 62-amino acid polypeptides containing: 20 cysteines, 6-8 lysines, 7-10 serines, a single acetylated methionine at the amino terminus. No aromatic amino acids or histidine are present. They are highly conserved and the large ratio of cysteine residues is responsible for the strong interaction of MT with a variety of heavy metal ions [9]. In addition, native MTs are heat-stable and highly resistant to proteolysis [12]. Metal binding also contributes to the stability of the molecule [13].

The possible functions of MTs are debatable. A role in heavy metal detoxification has been well documented, as has its function in the maintenance of zinc and copper homeostasis within mammalian cells [14]. An additional role for MTs appears to be protection against free radicals and radiation-induced oxidative stress [15, 16]. That MTs may serve as an expendable yet renewable free radical sink during hyper-oxygen exposure was hypothesized recently after evidence showed that preexposure of rats to Cd aerosols induced greater tolerance to hyperoxia. A 400-fold higher concentration of MTs was found in the lungs of animals pretreated with cadmium which may have protected the lungs from damage resulting from hyperoxia [17].

Both hsps and MTs exist in low levels in cells, and the syntheses of both are rapidly increased in response to stressful stimuli [7, 11]. For example, sodium arsenite, commonly used to induce synthesis of hsps [18], will also elevate MT levels [19]. Therefore, hsps and MTs are both inducible proteins known for their role in intracellular protection and aquired tolerance. Similarities between these 2 proteins led to the thesis that MTs are stress proteins.

Large differences between molecular weights (60-110 kDa vs. 5-7 kDa) of MT and hsps may have resulted in the failure to simultaneously detect these 2 proteins by standard techniques. In this study, a broad range electrophoretic separation was developed to resolve both hsp and MT on the same gel and methods for detection of MTs were established. For the induction of heat shock/stress response, sodium arsenite treatment was compared to heat shock treatment. Thermal stress was also used to examine possible intracellular response differences between chemical stress and thermal stress. Levels of both hsps and MTs in the study groups were compared and evaluated for their simultaneous induction.

MATERIALS AND METHODS

Cell Culture

HT29 cells, a continuous cell line derived from human intestinal epithelial adenocarcinoma, were obtained from the American Type Culture Collection (Rockville, MD) and used for these studies. Cells were maintained in Dulbecco's Minimal Essential Medium containing 10% fetal calf serum and subcultivated into Ambitubes or 6 well multiplates.

Cell Stress

Upon achieving confluence, the medium was changed and the cells were either treated with 75μM NaAr for 1 hour (n=6 cultures) or heat-shocked for 30 minutes at 43°C (n=6 cultures). Untreated cells (n=6) served as controls. At the end of the stress period, the cells were allowed to recover for an additional 3 hours at 37°C before radiolabeling.

Radiolabeling of Proteins

An hour prior to the end of the incubation period, cells were radiolabeled with [^{35}S] cysteine or [^{35}S] methionine (800 mCi/mmol, Amersham, Arlington Heights, IL) with 10 μCi/ml of radiolabel to quantitate the newly synthesized proteins. [^{35}S] methionine is commonly used to monitor protein synthesis but because MTs contain only small amounts of methionine, it may not be sufficiently labeled by this technique. Since the amino acid ratio of cysteine to methionine in MTs is 20:1, [^{35}S] cysteine was also selected as a tag for monitoring both hsps and MTs on the same gel. The incubation with the radiolabels was carried out either in cysteine-free or methionine-free medium for 1 hour at 37°C. At the end of the labeling period, the cells were rinsed once with balanced salt solution, and cells were lysed in a fresh solution of 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol, 12.5% 0.5M Tris-HCl buffer, 10% (v/v) glycerol and 0.025% bromophenol blue. Lysis was carried out until no intact cells could be seen by phase contrast microscopy (about 5 minutes). To reduce the viscosity of the cell extract, the lysates were forced through a 16 gauge needle. To completely denature proteins in the samples, the lysates were heated in double-boiler apparatus at 95-100°C for 5 minutes. After heating, the samples was centrifuged at 11,500 x g for 30 minutes to remove particulates.

Due to the presence of highly reactive thiols in MT, special precautions were needed to prevent its tendency for oxidation. These include storing samples at -70°C to prevent the protein from degradation, and use of fresh [^{35}S] cysteine to ensure successful radiolabeling of newly synthesized MTs.

Preparation of Beta Count of The [^{35}S]- Labeled Samples:

To estimate the amount of [^{35}S] cysteine or [^{35}S] methionine incorporated into proteins, a 5 µl aliquot of lysate from each sample was precipitated with an equal volume of ice-cold 10% trichloroacetic acid (TCA). The precipitate was collected onto a TCA-wetted 0.45µM nitrocellulose membrane, and rinsed dropwise with 1 ml of ice cold 10% TCA via suction filtration utilizing the millipore vacuum filter system (Millipore, Bredford, MA).

This step was important in removing any unbound [^{35}S] and preventing overestimates of the counts. The membrane was then air-dried and placed in a scintillation vial with 10 ml of Opti-Flour cocktail (ICN Biomedicals, Inc., Irvine, CA) for counting. A vial filled with 10ml of Opti-Flour cocktail was included with the experimental samples for determination of and correction for background counts. The total counts per minute (cpm) of each sample were determined by scintillation counting and used for determination of the sample loading volumes onto polyacrylamide gels prior to electrophoresis. Adjustment was made during loading of the samples such that each lane received an equal amount of TCA-precipitable radioactivity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system developed by Laemmli [20]. The 8-14% discontinuous vertical gradient gel seemed to work best for resolving the proteins of interest in this study. Tetramethylethylenediamine (TEMED), Tris HCl and Tris base, and Coomassie brilliant blue R-250 were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, glycine, bromphenol blue, and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Running gels contained 8%-14% acrylamide in 0.375 M Tris-Base, pH 8.8, with 0.01% SDS. Stacking gels contained 4% acrylamide in 0.5 M

Tris-HCl, pH 6.8, with 0.1% SDS. The gel solutions were filtered through 0.45-μm nitrocellulose filters, degassed, and chemically polymerized by the addition of TEMED and ammonium persulfate (3.3 ml 10% ammonium persulfate and 0.5 ml TEMED per liter running gel, 3 ml 10% ammonium persulfate and 1 ml TEMED per liter stacking gel). Stacking gels were 1.5 cm long and running gels were 13.5 cm long. The tank buffer consisted of 25 mM Tris buffer, with 0.1% SDS, and 1.5% glycine. Samples were lysed in buffer consisting of 5% 2-mercaptoethanol, 2% SDS, 12.5% 0.5M Tris-HCl and 0.025% bromophenol blue. Samples were loaded into gel lanes and separation of the proteins in one-dimensional SDS-PAGE is based on molecular weight. SDS in sample buffer eliminates charge variability between polypeptides, giving them all the same charge-to-mass ratio and forcing them into rod-like shapes, largely eliminating conformational and charge density variability. As proteins migrate through gradient gels, they encounter increasing acrylamide concentrations, hence the smaller pore size. Eventually, proteins reach the gel percentage where their migration is retarded by the gel pore size. The migration rate of individual polypeptides slows and almost stops, thereby tightening the bands. This produces distinct bands with good resolution and separation even with complex mixtures of proteins covering wide molecular weight ranges. The electrophoresis was carried out at 35 mA per gel with cooling (15°C) until the tracking dye reached the bottom of the gel. Gels were immersed in fixative (25% isopropyl alcohol and 10% glacial acetic acid) and agitated for 1 hour.

Protein bands were identified by staining with Coomassie brilliant blue R-250 (0.5 g per liter gel fixative) for at least 4 hours. Destaining was achieved by rinsing first in a 10% isopropyl alcohol and 10% glacial acetic acid solution for 1 hour followed by two 1 hour washes in 25% ethanol and 10% glacial acetic acid.

Estimation of the amount of newly synthesized protein was facilitated by fluorography. Gels were washed in isopropyl alcohol to remove excess water and impregnated with fluor (Amplify; Amersham, Arlington Heights, Illinois). Gels were soaked for 30 minutes in Amplify solution and then rinsed once with distilled H_2O. Gels were placed onto filter paper and dried at 80°C under vacuum for 2 hours (Bio-Rad slab gel dryer, Richmond, CA). Radiolabeled proteins in the gels were made visible with x-ray film (Kodak XAR-5, Rochester, NY). Films (Kodak X-Omat R) were pre-exposed to 0.1 optical density (OD) units using a camera flash. This procedure increased the sensitivity of fluorography and allowed for quantitative

analysis of the resulting fluorogram by correcting the non-linear relationship between radioactivity of the sample and absorbance of the film image [21] The film was then placed over the gel mounted into film cassettes (regular Kodak X-Omatic), exposed at -70°C for an appropriate exposure time, and then developed.

Estimation of molecular weights of proteins of interest were determined by reference to proteins of known MW (Rainbow Molecular markers, Amersham, Arlington Heights, IL) included with each gel run. Fluorograms were scanned with a laser densitometer (LKB Ultroscan XL, Bromma, Sweden) to quantitate the levels of stress proteins and MT present. The relative percent incorporation of each protein of interest, expressed as a percent of the total resolved radiolabel, was determined using software designed for the system.

Cadmium Binding for Identification of MTs

To confirm the identity of MTs on the SDS-PAGE gel, commercially available MTs from horse kidney were purchased from Sigma Co. These were horse MTs containing about 16 µg Zinc and 49 µg Cadmium per mg of MTs. Electrophoretic analysis often revealed impurities in these preparations with levels of contamination varying between lots. The MTs with the lowest contamination were then reduced in the fresh lysis buffer. This sample was heat treated at 100°C for 5 minutes, separated by SDS-PAGE using 4-20% Mini-PROTEAN II Ready gel (Bio-Rad, Richmond, CA), and electrophoretically transferred onto polyvinylidene difluoride (PVDF) immobilon membranes (Millipore Corporation, Bedford, MA) at a constant current of 100 mA for 1 hour with a Semidry trans-blot cell (Janssen, Piscataway, NJ). The method followed is described by Towbin et al. [22] in which 0.1% SDS was added to the transfer buffer to increase the efficiency of the protein transfer. Identification of MT was achieved by incubating the blot with 100 ml of Tris-HCl buffer containing 1 µCi/ml ^{109}Cd (Amersham 50-1000 µCi/µg Cd) for 15 minutes with gentle shaking as described by Aoki et al. [23]. MT retains its Cd-binding ability after separation on SDS-PAGE gels and electrophoretic transfer. After two 5-minute washes with deionized distilled water immediately following the Cd-incubation, the membrane was dried and placed in the film cartridge with intensifying screen and exposed at -70°C for an appropriate exposure time. The cadmium-binding proteins were visualized by autoradiography on X-ray film. After establishing the conditions for

positive binding potential of commercial MTs with ^{109}Cd, HT29 cytosols which had been treated as previously reported, with the exception of the radioisotope labeling step, were separated by the 4-20% ready mini-gels and tested for the presence of MTs.

RESULTS

ELECTROPHORESIS

Fig. 1 compares the pattern of proteins observed on SDS-PAGE gel stained with Coomassie brilliant blue R-250 with the fluorographic detection. Coomassie blue staining (Fig. 1A) indicated the total protein pattern and illustrated that relatively few changes in protein concentration occured over the course of the experiment.

The fluorogram in Fig. 1B illustrates alterations in protein synthesis during the course of the experiment. Proteins that undergo increased synthesis incorporate more radiolabeled amino acid and exhibit darker bands on the fluorogram. Proteins that are depressed in their synthesis exhibit fainter bands.

Fig. 2 compares the effect of heat shock and sodium arsenite on HT29 cell protein synthesis with respect to incorporation of [^{35}S] methionine and [^{35}S] cysteine. The pattern of the affected proteins in a representative fluorogram of an 8-14% SDS-PAGE reveals that 6 proteins have undergone substantial alteration in protein synthesis under the experimental treatment. Reference to molecular weight standards gives approximate molecular weight of 110, 90, 70, 32, 17 and 7.5 kDa. This protein migration pattern delimits the major members of heat shock proteins of hsp-110, 90, 70 and the heme oxygenase hsp-32 and MT. The identity of the protein with an approximate molecular weight of 17 kDa is unknown. Further analysis of the fluorogram shows that hsp-110, 90, 70, 32 and MT are also present at low levels in the control cells (Fig. 2, lanes A & D).

Heat shock led to increased synthesis of all major hsps (Fig. 2, lanes B & E) except hsp-32 (Fig. 2, lane E). Heat shock also caused a slight increase in the synthesis of MT protein (4 fold) (Fig. 2, lane B). Sodium arsenite caused induction of synthesis in all the major hsp protein. This treatment induced more intense hsp-70 (Fig. 2, lanes C & F) incorporation of methionine and also led to significantly increased synthesis of hsp-32 and MT, and decreased synthesis of a 17 kDa protein.

To quantitate the changes in the synthesis of proteins in response to heat shock and sodium arsenite, protein bands on fluorograms were scanned with a laser densitometer. Each line represents the scanned fluorographic image of a gel lane shown in the fluorogram, and each peak in each scan line corresponds to a

radiolabeled protein (band) in that lane. Because the fluorographic technique provides a linear response between incorporation of the radiolabeled amino acid in the sample and the image in the film [21], quantitative data are obtained. Comparison of these data indicates that [^{35}S] methionine-labeled samples gave the best representation of all the heat shock proteins (Fig. 5A & 5B). However, a broad band at 7.5 kDa was only observed in samples labeled with [^{35}S] cysteine. This phenomenon is to be expected, considering the high content of cysteine in MT and it provides good evidence for the identification of MT.

Comparing protein synthesis in response to the stress treatments with the untreated samples (Fig. 6) revealed that both heat shock and sodium arsenite seemed to have a similar effect on induction of hsp-110 and hsp-90 in HT29 cells. A greater induction of hsp-70 occurred after sodium arsenite treatment than after heat shock. There were large differences between heat shock and sodium arsenite induction of hsp-32 and MT synthesis in HT29 cell. These differences seem to be both qualitative and quantitative. Heat shock did not induce hsp-32 and had little effect on induction of MT (4-fold). In contrast, both MT and hsp-32 were induced by sodium arsenite (Fig. 8). The densitometric scans of fluorograms from sodium arsenite-treated cells that are labeled with either [^{35}S] cysteine or [^{35}S] methionine are displayed on the same scan to show the effects of sodium arsenite on induction of both hsp-32 and MT (Fig. 7). Sodium arsenite produced a 3-fold increase in hsp-32 and a 31-fold increase in MT compared to control (Fig. 9). The overall effects of both heat shock and sodium arsenite treatment in HT29 cells for the induction of all heat shock proteins including hsp-32 are summarized in Fig. 10. These values were pooled from triplicate [^{35}S] methionine samples. An analysis of these values indicated that although HT29 cells subjected to either thermal or sodium arsenite stress produced a 3-fold increase in both hsp-110 and hsp-90 syntheses compared to controls, heat shock produced a 4-fold increase in hsp-70 synthesis compared to controls. Sodium arsenite increased hsp-70 by 8-fold compared to control or about twice that of heat shock. Although heat shock did not induce hsp-32, sodium arsenite caused approximately a 2-fold increase in hsp-32 synthesis compared to controls (Fig. 11).

IDENTIFICATION OF MT THROUGH ^{109}Cd-binding

Although high [^{35}S] cysteine incorporation into MTs was a good indicator of its induction, an independent confirmation by

another method seemed desirable. Therefore, an attempt was made to utilize ^{109}Cd binding as evidence for the MTs located on the gel. MTs retain their metal-binding capability after denaturation and transfer [23]. To estimate the sensitivity of this assay, commercial horse MTs were separated by SDS-PAGE 4-20% mini-gel and western blotted onto a polyvinylidene fluoride (PVDF) membrane. Staining of the left-over gel with Coomassie brilliant blue indicated an approximately 50% transferring efficiency for the MTs. However, this transfer efficiency was not consistent between experiments (data not shown). The MTs transferred on the membrane were stained with amido-black 10B (E. Merck, Darmstadt, Germany), and the results are shown in Fig. 13, panel A as the image of multiple bands with 1 smeared band at 21 kDa and 3 sharper bands at lower MW level. This migration pattern was reproducible in separating the MT in this 4-20% mini-ready gel system, but was different from the cellular-induced MTs resolved on the 8-14% SDS-PAGE gel as reported previously. To assure the presence of MT band in the gel, this membrane was incubated with ^{109}Cd for 10 minutes and allowed to dry.

Detection for MTs in the membrane was accomplished by following the procedure stated in the Material and Methods section. The 21 kDa broad band was detected after a 1 hour exposure (Fig. 13, panel B, lane 1), whereas the other three bands were detected at 24 hours exposure (lane 2), all bands including molecular weight marker were detected after 87 hours of exposure (lane 3). This result indicated that the 21 kDa protein was MT in this mini-gel system. Cold samples of HT29 cells, treated as previously reported, were separated on the 8-14% gel, immediately transferred onto the blot membrane, and tested for the presence of MT with the cadmium-binding assay. The 24 hour exposure was attempted first, but yielded a negative result. Both the low concentration of induced MTs and the low transfer efficiency of this protein were suspected as contributing factors for failure of this assay. With extended exposure, many Cd-binding protein bands, including some of the molecular weight standard proteins, began to show on the membrane (Fig. 14). Therefore, the specificity of this assay for identification of MTs in a complex mixture of proteins was limited.

DISCUSSION

Although hsps or stress proteins and MTs play a role in the normal economy of the cell, they are preferentially stimulated to high levels after sublethal injury. These high levels are thought to provide a measure of protection against further changes in the environment. The similarity of hsps and MTs with respect to inducibility and protection from sub-lethel injury [1, 9] stimulated this study to examine the relationship between MTs induction and the heat shock response.

Heat shock is the classic method for inducing heat shock proteins [1]. Sodium arsenite has also been used to study the induction of heat shock proteins [24], and sodium arsenite also induces MTs in vitro [19]. This study is the first to show simultaneous induction and detection of both hsps and MTs in human cells by either heat shock or sodium arsenite stress by SDS-PAGE.

Comparison of the synthesized proteins after [^{35}S] methionine and [^{35}S]cysteine labeling of HT29 cells exposed to either heat shock or arsenite revealed 3 qualitative and quantitative differences between thermal stress and sodium arsenite. These differences were: (1) Although both heat shock and arsenite stress induced a common set of major heat shock proteins (hsp-ll0, hsp-90 and hsp-70) in HT29 cells, the intensity of the induction is stronger in hsp-70 protein synthesis after sodium arsenite treatment. Arsenite induced twice as much hsp-70 as heat shock. Slow elimination of arsenite from the cell after removal from heat shock may explain this difference [1]. The type of stressing agent had no effect on the levels of hsp-110 and hsp-90. (2) In addition to the common hsps induced by both stresses, arsenite also induced a 32 kDa hsp (heme oxygenase) and MTs. Heat shock did not induce the heme oxygenase, but led to a small elevation of MTs. (3) Arsenite, but not heat shock, strongly depressed synthesis of a 17 kDa protein.

The induction of MTs was evident in a 7.5 kDa band which could be seen after heat shock or arsenite treatment on the 8-14% SDS-PAGE gel after [^{35}S] cysteine labeling. Attempts were made using ^{109}Cadmium binding assay, but the presence of other Cd-binding proteins in cell extracts made the detection of MT difficult. Immunochemical methods to identify this protein are currently underway. The presence of MTs is strongly suggested by 4 pieces of information which support our conclusion: 1) The characteristically

diffused MT band pattern displayed on the SDS-PAGE gel agrees with the MT band pattern reported by others [25]. 2) This protein migrated to the expected molecular weight position on the 8-14% SDS-PAGE gel. 3) This protein appeared in [^{35}S]cys-labeled samples, but was not visible in [^{35}S]met-labeled samples. Thus, this protein contains little methionine, but is comparatively high in cysteine. Since MTs contain only one methionine; 20 cysteine, this result is predictable. 4) As expected of MT [19], the bands underwent tremendous increases (31-fold) in synthesis following treatment with arsenite. The same band underwent slight (about one-tenth that of arsenite-treated) increases in synthesis following heat shock.

Two other proteins might be confused with MTs in the SDS-PAGE gel under our experimental conditions. They are ubiquitin and the MT transcription factor. Ubiquitin is a small molecular weight (8.5 kDa) heat shock protein that has not previously been observed on standard gels [1]. Analysis of its amino acid sequence revealed that ubiquitin contains many methionine molecules, but has no cysteine [26]. Exclusive radiolabeling of the 7.5 kDa protein with [^{35}S]-cys revealed no presence of ubiquitin in that band. The MT transcription factor is a 225 amino acid long protein with a high content of cysteine (reviewed by Andrews [27]) and therefore would be detected in a higher MW range. The absence of the MT transcription factor thus rules out its being mistaken for MTs. The complete list of intracellular proteins which can be detected by two dimensional electrophoresis SDS-PAGE [28] indicates that no other proteins in this MW range can be observed after labeling with either [^{35}S] methionine or ^{14}C amino acid.

The function of the polypeptides involved in this response is under intensive investigation. Protection of cells against subsequent, otherwise lethal thermal stress was first proposed as the major function for heat shock proteins [7], whereas metal detoxification was the first function suggested for MTs [11].

Little information is available about Hsp-110 (100-110 kDa). Hsp-110 is a constitutively expressed protein whose synthesis can be elevated by heat. This protein is found in the nucleolus and may be involved in rRNA transcription (reviewed by Welch *et al.* [29]).

Hsp-90 (83-90 kDa) is relatively abundant in unstressed cells, but is more strongly induced by heat [7]. It has been isolated from the cytosolic fraction of the cells and is not found in the nucleus [30, 31].

Neither the mechanisms of its induction nor its physiological functions are fully understood. All steroid hormone receptors in vertebrates form inactive complexes with hsp-90 from which they can be dissociated by the hormone, high salt, or metal chelators (reviewed by Lindquist and Craig[7]). It has been hypothesized that receptors bind to the highly charged region near the amino terminus of hsp 90 to form an inactive complex, and that the subsequent binding of hormone to receptor initiates the release of hsp 90 [32]. Hsp 90 has also been associated with several protein kinases [33]. The ability of hsp 90 to interact with other proteins has led to its inclusion as a molecular chaperone [34].

Hardesty and Kramer proposed [35] that potentially active components of proteins such as steroid receptors or tyrosine kinases are bound at specific sites along the elongated hsp 90 protein in an inactive state and then released when needed. The release of the specific component is promoted by special initiating events such as heat shock, binding of a steroid hormone, or phosphorylation.

Hsp-70 (68-73 kDa) is the most thoroughly studied stress protein family. There are two major members in this family: the 73 kDa protein which is constitutively synthesized but is not elevated by heat shock, and the 72 kDa protein which increases only after induction of the stress response [36]. Hsp-70 exhibits a high affinity for ATP [37] and is found in both nucleus and cytoplasm [38]. Hsp-70 plays an important role in translocation of secretory and mitochondrial precursor proteins from their site of synthesis in the cytoplasm across the endoplasmic reticulum into mitochondria. This translocation activity is ATP-dependent [8]. Hsp70 also seems to be involved in the correct folding of proteins in normal cells [34], and was found to solubilize denatured proteins by an ATP- dependent process of a repeated cycle of binding and releasing of the protein aggregates [39].

Hsp-32 (30-35 kDa) is found only in the cytoplasm [40]. It has now been identified as heme oxygenase [41], a microsomal membrane protein that catalyzes the oxidative degradation of heme to biliverdin, a precursor protein of a strong antioxidant named bilirubin. The precise molecular weight of hsp-32 depends on the species of origin. In human and rat, it is 32 kDa, and in mouse it is 34 kDa [42]. The induction of this protein in cells was initially thought to be limited to inducing agents that affect sulfhydryl groups such as heavy metals. However, later studies have shown that ultraviolet light and H_2O_2 also lead to induction of this protein,

suggesting that hsp-32 may play a role in the defense against oxidative stress, i.e., stress initiated by uncontrolled oxidation. It was hypothesized that hsp-32 may protect directly against oxidative damage in normal human skin cells. Hsp-32 is not induced by heat shock in humans [43], but is induced by heat shock in rat hepatoma cells [44].

MTs (7-10 kDa) are cytoplasmic polypeptides with a high content of cysteine which predisposes them to form coordination complexes with heavy metal. All vertebrates examined contain two or more distinct MT isoforms which fall into one of two classes, designated MT-I or MT-II, depending on the elution position from the gel filtration column [11]. MTs are induced by heavy metals, glucocorticoids, cytokines [45], UV light [15], bacterial endotoxin, alcohol, and X-ray irradiation [45]. MTs are found in all cells which have been studied [14]. Besides their function in metal detoxification and the maintenance of cellular zinc and copper homeostasis [9], there is evidence that MTs also play an important protective role against free radical and radiation-induced oxidative stress [15, 16]. MTs have recently been denoted as proteins induced by oxidative stress [46].

The identity of the major hsps induced by both heat shock and arsenite was demonstrated by using the method of peptide mapping. Pairs of major hsp, e.g., hsp-70 induced by both heat shock and arsenite, were digested with a protease. The digested protein mixture was then analyzed with two-dimensional SDS-PAGE. The same values of molecular mass and isoelectric point were obtained [47]. The 32 kDa protein induced by UVA, H_2O_2 and NaAs was also found to be closely related and possibly identical by partial peptide mapping [43]. It was therefore concluded that heat shock proteins induced by different agents are structurally identical and not unique to each inducer [47]. The molecular events associated with heat shock and arsenite-induced stress responses were also investigated by Amaral et al. Using actinomycin D (a transcription inhibitor that blocks RNA synthesis) and cycloheximide (an inhibitor molecule that blocks the protein translation) Amaral et al. suggested that both heat shock and arsenite-induced response are mainly controlled at the transcriptional level for the major hsps and at both transcriptional and translational levels for small molecular weight proteins including hsp-32.

The mechanism(s) involved in heat shock and oxidative-induced stress response is still controversial. Abnormal proteins,

unfolded proteins and proteins damaged by free radicals have been proposed to trigger both stress responses. The simultaneous induction of hsps and oxidation-specific stress proteins suggests a common pathway exists for their induction, but the specific synthesis of a subset of oxidative stress proteins such as heme oxygenase argues for separate pathways regulating the induction of heat shock proteins and oxidation-specific stress proteins [46].

A common induction mechanism, however, has been challenged. The strongest attack on a common mechanism for induction came from Maytin and Young. Using glucocorticoid (dexamethasone), cadmium and heat shock as three separate agents, their study revealed the induction of non-overlapping sets of gene products in thymic lymphocytes of rats in vivo. "In no case was a protein found to be inducible by more than one agent." [48] They hypothesized an agent-specific stress protein induction mechanism with each agent initiating its own specific response through a separate gene-induction mechanism. Limitation of protein separation techniques has been cited as the cause of precluding detection of the full range of responses to the other inducing agents [48].

Since the thesis was formulated that MTs are hsps, considerable progress has been made in the regulation of both hsps and MTs. The induction of hsps in response to heat shock is initiated by the binding of a trans-acting heat shock factor (HSF), to a short, highly conserved DNA known as the heat shock element (HSE). In mammalian cells, HSF preferentially forms large complexes, and only HSEs containing extended nGAAn arrays activate transcription when located far from the TATA box. These results imply that activated HSE contains multiples of HSF monomers. Hsps are suggested as regulators of HSFs. These hsps might regulate HSF in unstressed conditions by binding to the monometric HSF unit. Among many signal proteins proposed, abnormal protein was suggested as the most possible cause for triggering this event [18]. During heat shock, high levels of abnormal proteins which have been damaged by heat compete with the hsps to bind the HSF unit which leads to the dissociation of HSF-hsp complexes and facilitates the assembly of HSF into oligomeric complexes during induction, and returns HSF to an inactive configuration during recovery (reviewed by Sorger [49]).

The gene promoters of MTs are multiple cis-acting DNA sequences involved in basal, as well as inducible, gene transcription.

These DNA sequences, termed metal-response-elements (MREs), are binding sites for trans-acting transcription factors that regulate the level of transcription of the MT genes in response to stimuli such as metal concentrations. In humans, the MT genes possess multiple (5 or more) MREs, most of which are located within the 100-bp region of the promoter upstream of the TATA box [50].

Similar trans-acting transcription factors are also involved in MT gene transcription. ACE 1, a trans-acting transcription factor from the yeast Saccharomyces cerevisiae has been cloned, and the mechanism of copper (Cu) regulated transcription in yeast has been investigated in detail [27]. The ACE1 gene codes for a transcription factor ACE1 that, in the presence of Cu, is activated by the cooperative binding of Cu to form a binding complex. This complex binds to multiple copies of an upstream activation sequence of the Cu-thionein gene promoter. High levels of MT gene transcription then occur [51, 52]. The regulation of yeast Cu-thionein suggests that metal regulation of transcription of MTs represents a simple positive regulatory circuit involving metal ions, the ACE1 transcription factor protein, and the upstream activation sequence of the metal-thionein promoter [27].

MTs have also been proposed to be induced independent of metal binding by free hydroxyl (·OH) and superoxide (O_2^-) radicals [15, 53]. Recently, a single binding site for ACE1 transcription factor was found in the superoxide dismutases SOD1 promoter region of yeast [53, 54]. Superoxide dismutases (CuSOD, ZnSOD) are metalloproteins that help minimize oxygen toxicity. They catalyze the dismutation of superoxide.

$$2\,O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2 \qquad [55].$$

This finding suggests that ACE1 not only regulates MTs, but also controls the level of SOD1 at the transcriptional level.

SOD and hsp-32 share many similarities. Like SOD, Hsp-32 is a metalloprotein with antioxidant functions [41]. Greater amounts of hsp-32 increase cellular capacity to generate biliverdin by catalyzing the oxidative degradation of heme to biliverdin. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Unconjugated bilirubin is an efficient scavenger of reactive oxygen [56] and is able to react with superoxide anion and peroxyl radicals [57].

Numerous studies have shown that strong MT inducers such as most transition series metals (Zn, Cu, Cd) and arsenite [9], cause persistent induction of hsp-32 in eukaryotic cells [42, 47]. "In fact, expression of this protein is a uniform response to cellular challenge with arsenite and no exception has been identified to date" [40]. The failure to realize that both proteins are coinduced probably resulted from the method of protein separation and detection used in the study of hsp-32. The antioxidant functions of both hsp-32 and SOD suggest an MT-SOD gene regulatory relationship may occur between MTs and hsp-32. The existence of an ACE1 binding site in the promoter region of hsp-32 is possible. However, this hypothesis remains to be tested.

Although all MTs gene promoters contain metal responsive elements (MREs) to regulate the level of transcription of the MT gene in response to metal concentrations, other responsive elements such as glucocorticoid responsive elements (GRE) [58], interferon responsive elements (IRE) [59], and heat shock responsive elements (HSRE) [60] have recently been defined in a few MT gene promoters as well. These responsive elements represent binding sites for transcription factors that are known to interact with promoters from many genes, and may interact with other inducible elements and enhance basal levels of gene transcription [27].

The presence of other response elements on MT gene promoter region is apparent in recent work by Iijima et al. In this study, MTs were found synergistically induced by either a cytokine or a heavy metal with a glucocorticoid hormone and were additively induced by the combination of a cytokine and a heavy metal in human liver cells. The relationship of MT induction to the heat shock response may belong to this category of regulation [45].

The possibility of synergistic induction of MTs with both heat shock and arsenite is unknown. The ability of low efficient binding of the HSF to the MRE (CUP1) region of MT DNA [61] offers an alternative explanation for the low level of MT induction observed during heat shock in this study. The detection of this low level MT synthesis under heat shock treatment, as demonstrated in this study, is probably based on the sensitivity of the protein separation and detection method used.

In conclusion, MT is a stress protein and also an hsp because its synthesis can be induced by heat shock. The syntheses of hsps and MT can be induced simultaneously by either heat shock or

sodium arsenic in HT29 cells. MTs and hsp-32 are both coinduced by sodium arsenite, but hsp-32 is not induced by heat shock. The induction of MTs by heat shock was low, relative to sodium arsenite. This indicates that MT induction is regulated independently from the induction of hsps.

Future studies should investigate possible synergistic induction of heat shock proteins and MTs. It may be also possible to find a physiologically compatible inducer to achieve a synergistic protective effect of both stresses with minimum cellular toxicity.

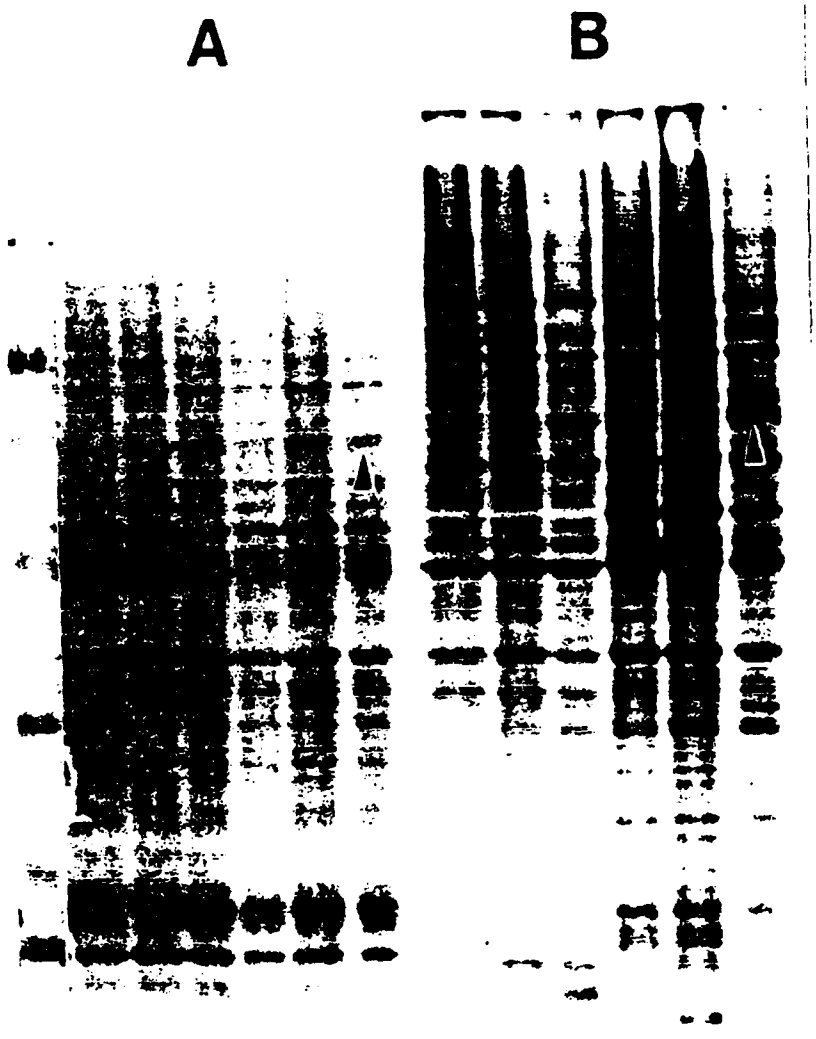

Fig. 1. Comparison of coomassie blue staining vs fluorographic detection of proteins. HT29 cells were either heat shocked at 43°C for 30 min. or exposed to 75μM sodium arsenite at 37°C for 1 hr. A, wet SDS-PAGE gel stained with coomassie brilliant blue R-250 which indicates total protein concentration. B, fluorogram of the gel shown in A to demonstrate alterations in protein synthesis after experimental treatment. Proteins that undergo increased synthesis incorporate more radiolabeled amino acid, and appear darker on the fluorogram. A single lane of molecular weight standard that comigrated with the cellular proteins is indicated on the left.

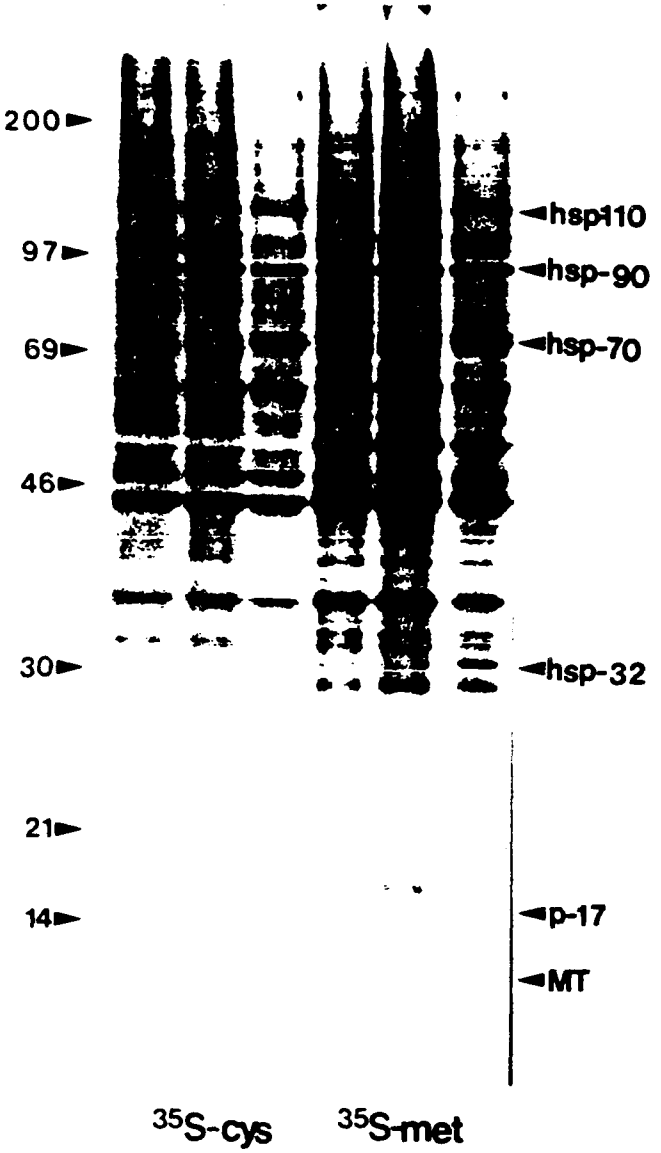

Fig. 2. Effects of heat shock (43°C) and sodium arsenite (75μM) on HT29 cells detected by fluorography.

- A Control cells radiolabeled with [^{35}S] cysteine
- B Heat shock treated at 43°C and radiolabeled with [^{35}S]cysteine.
- C 75μM sodium arsenite treated and radiolabeled with [^{35}S] cysteine.
- D Control radiolabeled with [^{35}S] methionine.
- E Heat shock treated at 43°C and radiolabeled with [^{35}S] methione.
- F 75μM sodium arsenite treated and radiolabeled with [^{35}S] methionine.

Proteins were separated on an 8%-14% gradient SDS polyacrylamide gel. Arrows indicate stress proteins, MTs are on the right, and molecular weight standards are on the left.

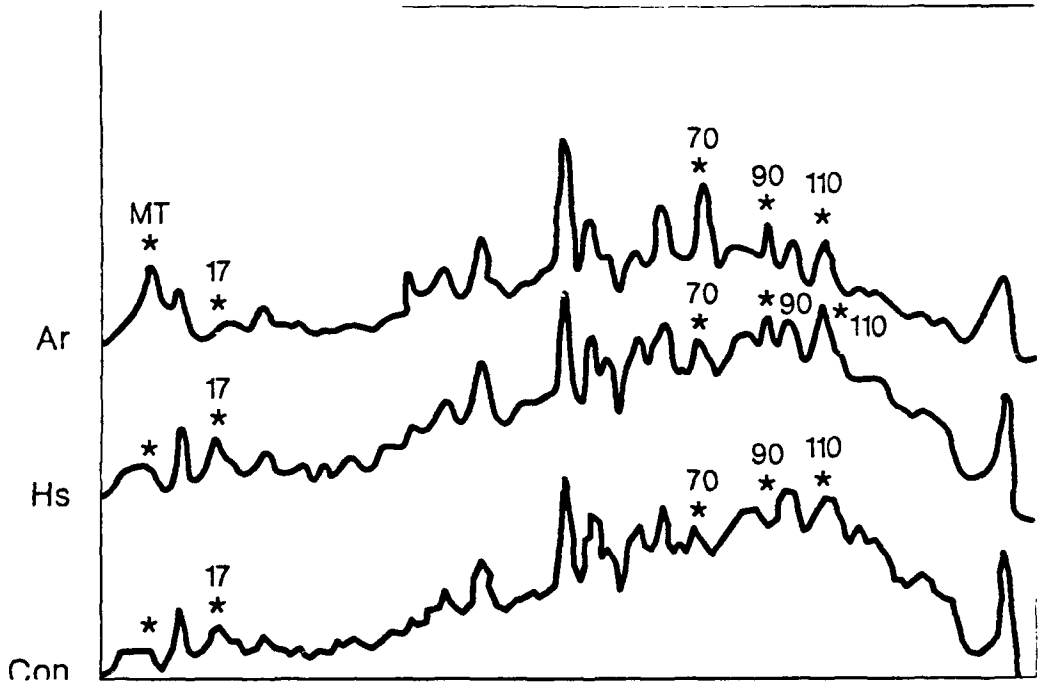

Fig. 3. Laser densitometric scan showing the effect of heat shock (43°C) and sodium arsenite (75μM) on incorporation of [^{35}S]. This is a scan of a representative fluorogram. The samples here are exclusively radiolabeled with [^{35}S] cysteine. The three scan lines are the fluorographic image of the gel lanes in 2; A, control; B, heat shock (43°C), C, sodium arsenite (75μM). Each peak corresponds to a radiolabeled protein band in that lane. Proteins that underwent alteration in synthesis are marked with (*) here.

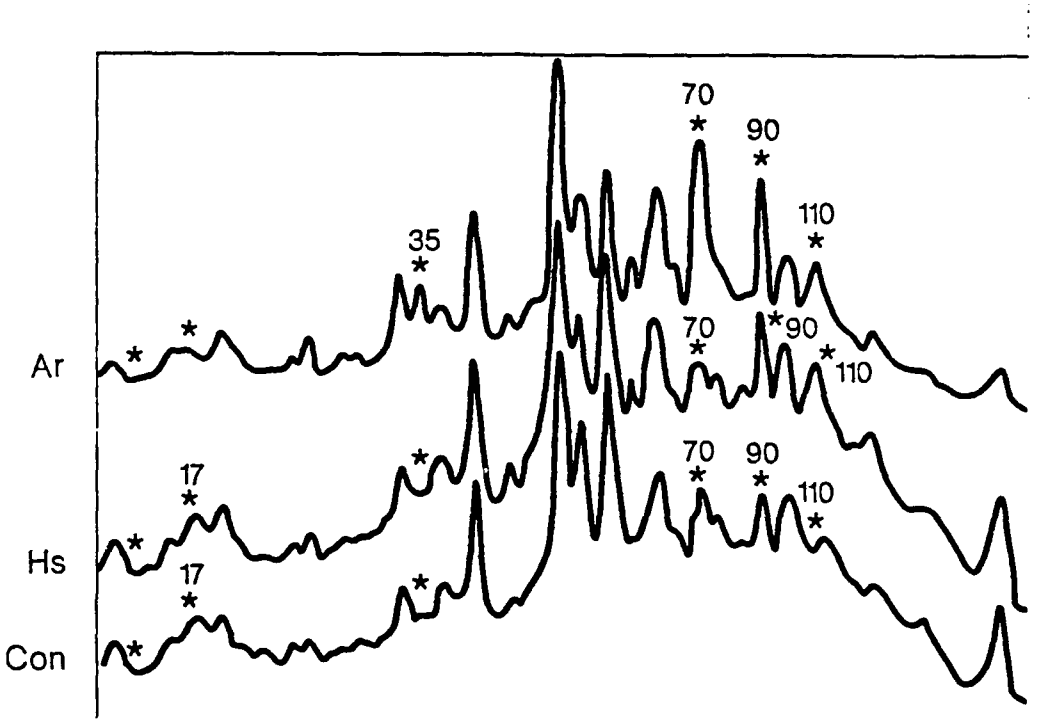

Fig. 4. Laser densitometric scan showing the effect of heat shock (43°C) and sodium arsenite (75μM) on [^{35}S] methionine incorporation. This is a scan of a representative fluorogram. The three scan lines presented here are the fluorographic image of the gel lanes in Figure 2. Lane D, control; lane E, heat shock (43°C); lane F, sodium arsenite (75μM). Each peak corresponds to a radiolabeled protein band in that lane. Proteins that underwent alteration in synthesis are marked with (*) here. The samples here are exclusively radiolabeled with [^{35}S] methionine. The absence of the marked peaks,and the variation in amplitude of the peaks reflects the effects of both stresses on the cells.

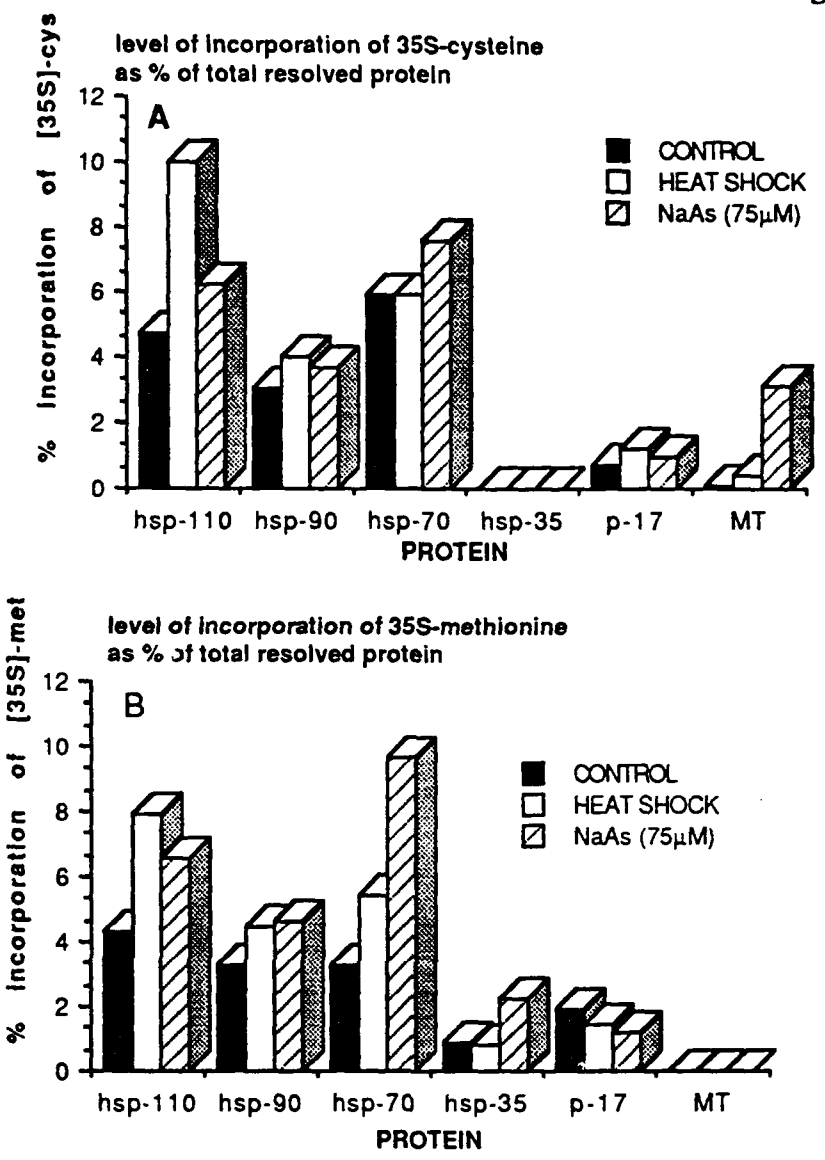

Fig. 5 Changes in heat shock proteins and metallothioneins synthesis following heat shock or sodium arsenite treatment. A. Samples labeled with [^{35}S] cysteine; B. Samples labeled with [^{35}S] methionine.

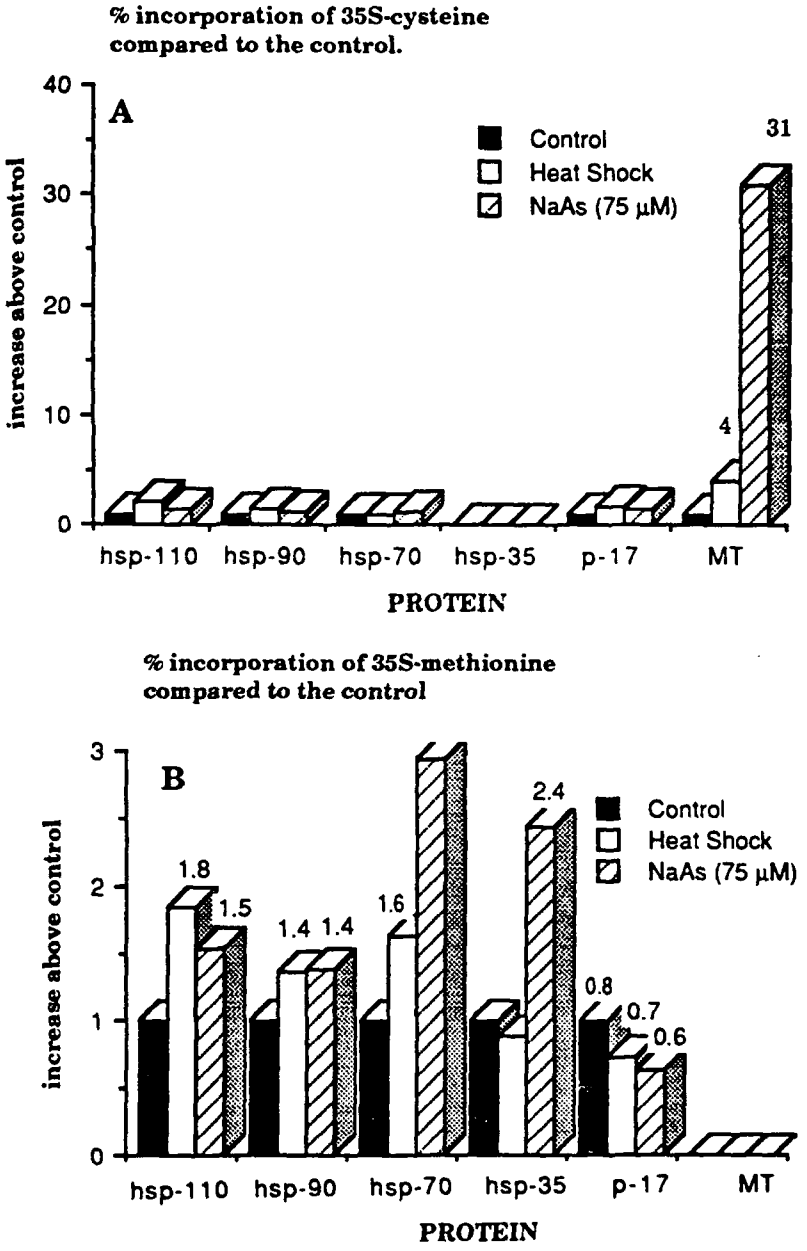

Fig. 6: Changes in heat shock protein and metallothionein syntheses compared to control following heat shock or sodium arsenite treatment. A. Samples labeled with [^{35}S] cysteine; B. Samples labeled with [^{35}S] methionine.

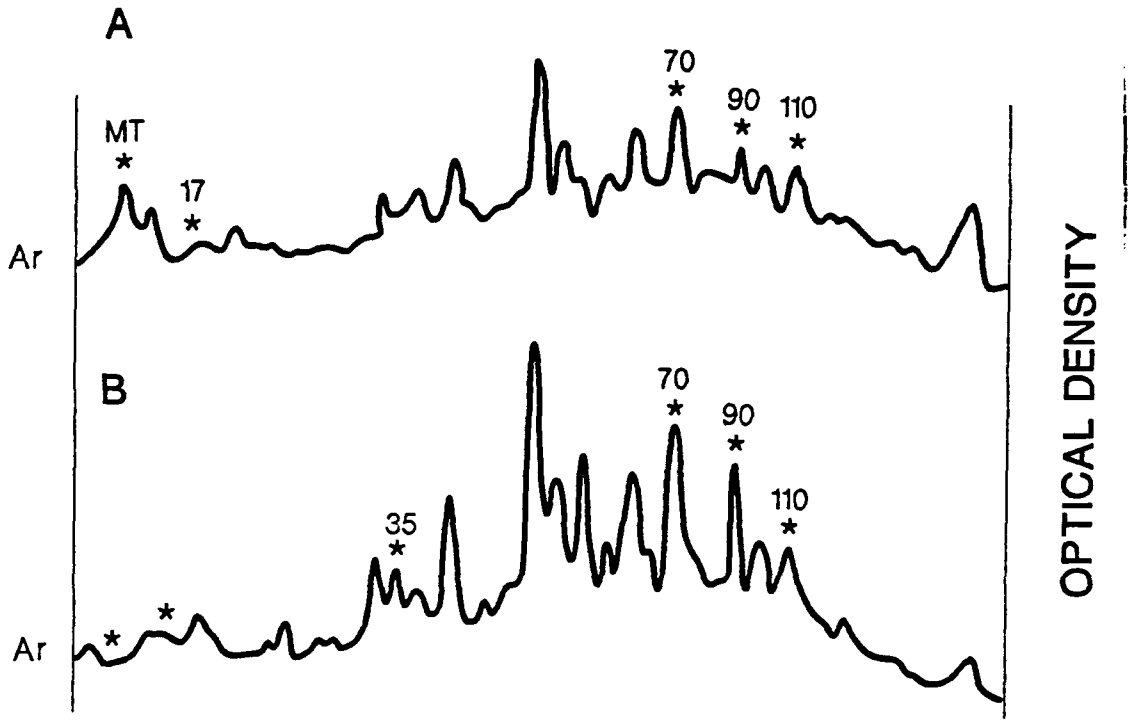

Fig. 7. Laser densitometric scan showing the effect of sodium arsenite (75 μM) on induction of hsp-32 and MTs in HT29 cells. The two scan lines presented here are the fluorgraphic image of the gel lanes in Figure 2. lane C, sodium arsenite (75μM), Samples labeled with [^{35}S] cysteine; lane F, sodium arsenite (75μM), samples labeled with [^{35}S] methionine. Each peak corresponds to a radiolabeled protein band in that lane. Proteins that underwent alteration in synthesis are marked with (*) here. The absence of the marked peaks,and the variation in amplitude of the peaks reflects the effects of sodium arsenite on the cells.

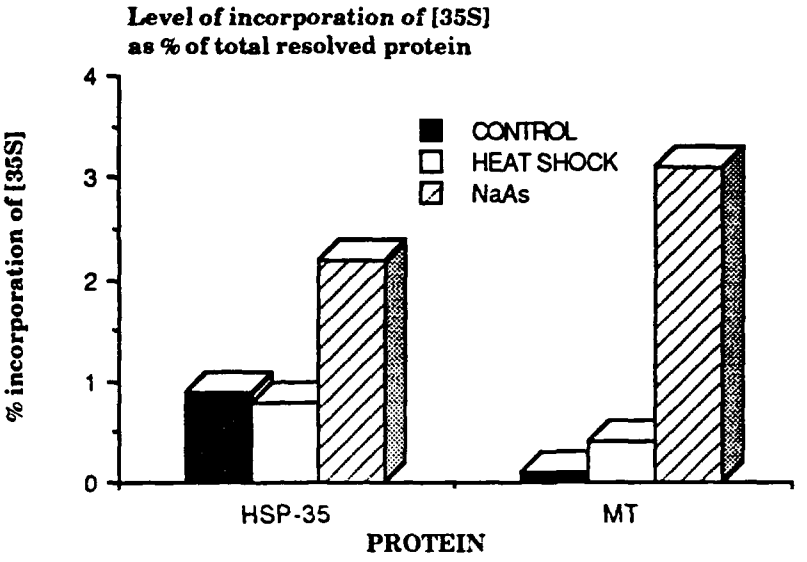

Fig. 8. Changes in hsp-32 and metallothioneins synthesis following sodium arsenite treatment. Hsp-32, samples labeled with [^{35}S] methionine; MTs, samples labeled with [^{35}S] cysteine.

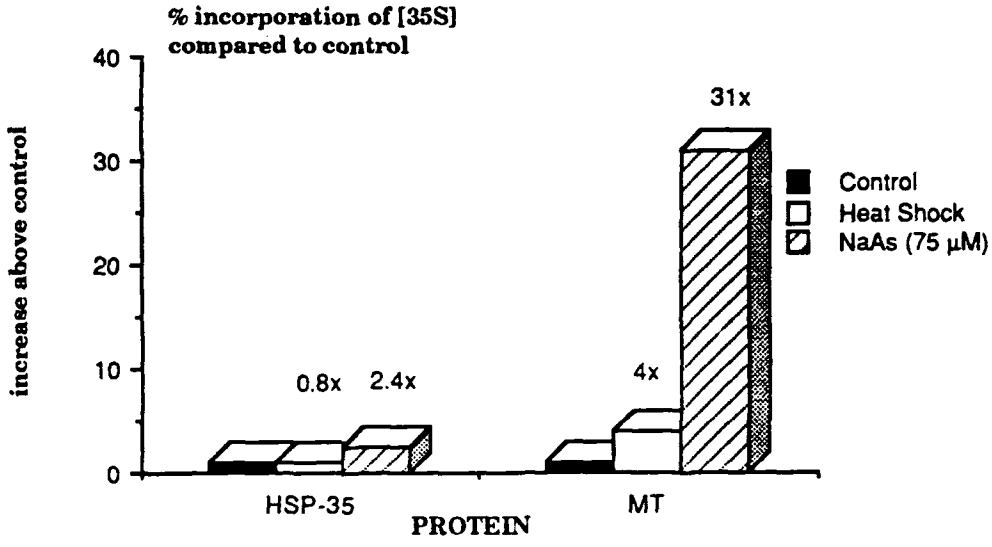

Fig. 9. Changes in hsp-32 and metallothioneins synthesis compared to control following sodium arsenite treatment. Hsp-32, samples labeled with [^{35}S] methionine; MTs, samples labeled with [^{35}S] cysteine.

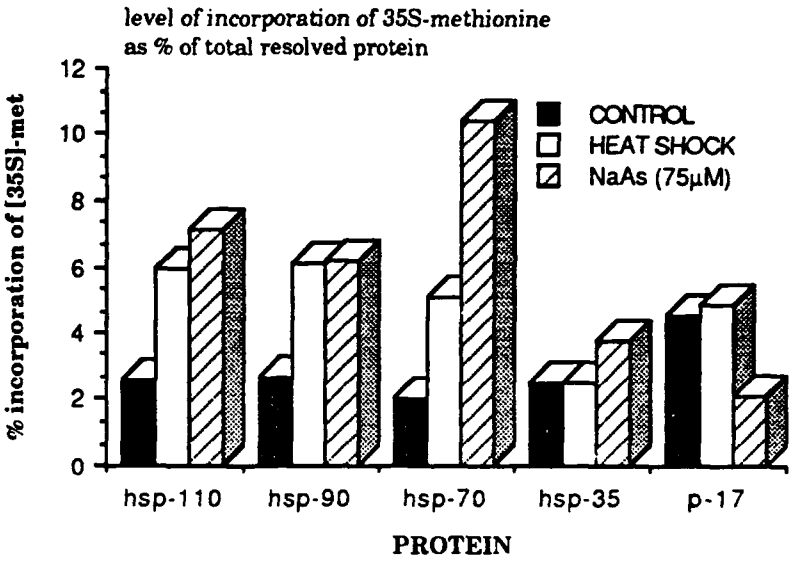

Fig. 10. Changes in heat shock proteins synthesis following heat shock or sodium arsenite treatment. Graph presents average data values from triplicate samples labeled with [^{35}S] methionine.

1 2 3

Fig. 11. Evidence of specific ^{109}Cd binding with commercial MTs.

lane 1, MW; lane 2, amido-black stained blot; lane 3, 1hr ^{109}Cd-MTs exposure. Proteins were separated on a 4-20% mini-ready gel and transfered onto the membrane. The band diffuses and has high affinity for ^{109}Cd.

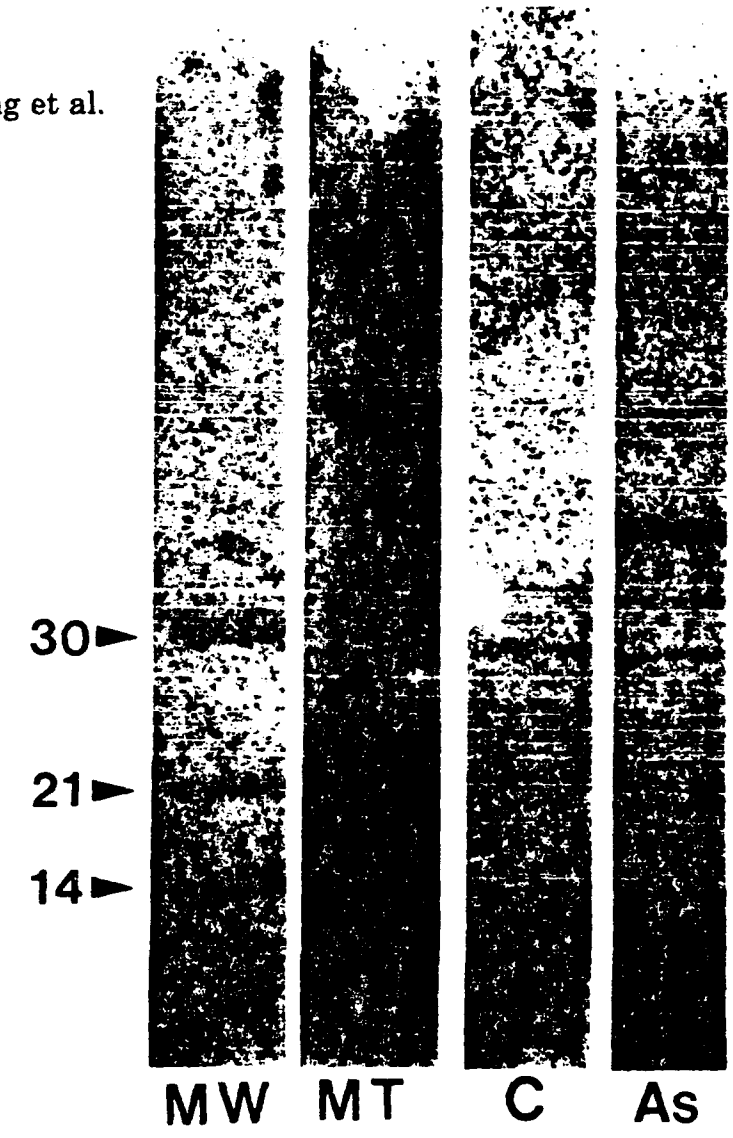

Fig. 12. Evidence for ^{109}Cd-binding with other proteins in cell extracts; Lane 1, MW markers; lane 2, MTs standard; lane 3, control; lane 4, sodium arsenite (75μM). Proteins were separated on a 4-20% mini-ready gel and transfered onto the membrane. All lanes were exposed for 3 days.

Table 2. Changes in incorporation of [35S] cysteine following thermal stress or sodium arsenite exposure presented as percent of total in HT29 cells (proteins were separated on 8-14% SDS-PAGE gradient gel).*

Protein	Control	Heat Shock	NaAs (75 μM)	ctl/ctl	HS /ctl	As/ctl
hsp-110	4.70	10.00	6.20	1.00	2.13	1.32
hsp-90	3.00	4.00	3.70	1.00	1.33	1.23
hsp-70	5.90	5.90	7.60	1.00	1.00	1.28
hsp-32	0.00	0.00	0.00	0.00	0.00	0.00
p-17	0.70	1.20	1.00	1.00	1.71	1.43
MTs	0.10	0.40	3.10	1.00	4.00	31.00

* The optical density and band width of each protein's fluorographic image was measured using a scanning densitometer. The OD X mm values were then summed for each lane. Thus, the OD X mm value for each individual protein was responsible for some percentage of the lane's total. Changes in percentage from lane-to-lane are therefore directly proportional to alterations in incorporation of radiolabel.

Table 3. Changes in [35S] methionine incorporation following thermal stress or sodium arsenite treatment presented as percent of total in HT29 cells. (proteins were separated on 8-14% SDS-PAGE gradient gel, data from triplicate determinations).*

Protein	Control	Heat Shock	NaAs (75 μM)	Ctl/Ctl	HS/Ctl	As/Ctl
hsp-110	2.57	6.00	7.13	1.00	2.63	3.40
hsp-90	2.67	6.10	6.17	1.00	4.48	4.45
hsp-70	2.00	5.13	10.47	1.00	3.36	7.75
hsp-32	2.50	2.50	3.75	1.00	0.96	1.87
p-17	4.57	4.90	2.13	1.00	1.00	0.50

* The optical density and band width of each protein's fluorographic image was measured using a scanning densitometer. The OD X mm values were then summed for each lane. Thus, the OD X mm value for each individual protein was responsible for some percentage of the lane's total. Changes in percentage from lane-to-lane are therefore directly proportional to alterations in incorporation of radiolabel.

ACKNOWLEDGMENTS

We would like to express our appreciation to Dr. Evelyn McGown of the Blood Research Division for her helpful discussions, and Andre Akers and Wally Wong for photographic assistance. In addition, we thank Dr. Crellin Pauling and Dr. Sarane Bowen for serving as graduate committee advisors.

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